

## (12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

ONTVANGEN

CORRECTED VERSION

9 DEC 2002

GIANT  
(19) World Intellectual Property Organization  
International Bureau(43) International Publication Date  
25 July 2002 (25.07.2002)

PCT

(10) International Publication Number  
WO 02/057779 A2

(51) International Patent Classification: G01N 33/50 (81) Designated States (national): AE, AG, AL, AM, AT (utility model), AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU (inventor's certificate), CZ (utility model), CZ, DE (utility model), DE, DK (utility model), DK, DM, DZ, EC, EE (utility model), EE, ES, FI (utility model), FI, GB, GD, GE, GH (utility certificate), GM, HR (consensual patent), HU, ID, IL, IN, IS, JP, KE, KG, KP (inventor's certificate), KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK (utility model), SK, SL, TJ, TM, TN, TR, TT (utility certificate), TZ, UA, UG (utility certificate), US, UZ, VN, YU, ZA, ZM, ZW.

(21) International Application Number: PCT/NL02/00039

(22) International Filing Date: 18 January 2002 (18.01.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data: 01200181.4 18 January 2001 (18.01.2001) EP

(61) Related by addition to earlier applications or grants:  
CU Not furnished (ICA)  
Filed on Not furnished  
KP Not furnished (ICA)  
Filed on Not furnished

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WO 02/057779 A2

(84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

## Published:

— without international search report and to be republished upon receipt of that report

## (48) Date of publication of this corrected version:

21 November 2002

## (15) Information about Correction:

see PCT Gazette No. 47/2002 of 21 November 2002, Section II

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

Kopie inleveren	TERMIJN	18-07-2002 (quar.)
	= 4 DEC 2002	
	GEANTWOORD	
	MAP P54974 PCou	

(54) Title: CLONING AND EXPRESSION OF A NEW MCP RECEPTOR IN GLIAL CELLS

(57) Abstract: The invention relates to the fields of inflammation and immunology, and more specifically to the field of chemokines and receptors therefor, and their role in neurodegenerative or neuroinflammatory disease. The invention provides a method for identifying a candidate drug compound for the treatment of inflammatory or degenerative brain disease comprising testing said compound for its capacity to modulate or mimic MCP-1 binding with a chemokine receptor capable of being expressed on brain glial cells, said receptor known in the mouse as L-CCR or in humans as CRAM-B.



## Title: Cloning and expression of a new MCP receptor in glial cells

5 The invention relates to the fields of inflammation and immunology, and more specifically to the field of chemokines and receptors therefore, and their role in neurodegenerative or neuroinflammatory disease.

10 Chemokines are small chemotactic cytokines of approximately 10kDa, which orchestrate the inflammatory response by attracting leukocytes to sites of inflammation and by controlling the homing of dendritic cells, T cells and B cells (for review see: 1, 2, 3). Chemokines and their receptors, all of which are G-protein coupled, are subdivided into four families: CXC-, CC-, C- and CX3C-chemokines (3). Chemokine signaling is highly promiscuous, most chemokines activate more than one chemokine receptor and vice versa (4, 5). In humans more than 25 CC chemokines and 10 CC chemokine receptors (CCR) have been cloned (3). Furthermore, it is likely that some of the currently known orphan chemokine receptors will make chemokine signaling even more complex (4).

15 Ishizuka et al.: Identification of monocyte chemoattractant protein-1 in senile plaques and reactive microglia of Alzheimer's disease (Psych. Clin. Neurosci. 1997) stress the importance of the chemokine MCP-1 in the pathology of Alzheimer's disease.

20 However, no mention of involvement of specific chemokine receptors has been made.

Also, Berman et al.: Localization of Monocyte Chemoattractant Peptide-1 Expression in the Central Nervous System in Experimental Autoimmune Encephalomyelitis and

25 Trauma in the Rat (J. Immunol. 156, 1996) shows expression of the chemokine MCP-1 in experimental autoimmune encephalitis (EAE), a model for MS. From experiments using MCP-1 knock out mice the importance of MCP-1 in EAE pathology is now well established. The MCP-1 receptor currently considered important by Bermann et al is CCR2, the publication thus focuses on MCP-1 and CCR2 knock out mice. Koch et al.: Enhanced Production of Monocyte Chemoattractant Protein-1 in Rheumatoid Arthritis

30 (J. Clin. Invest. 1992) again clearly supports a role of the chemokine MCP-1 in rheumatoid arthritis and the subsequent chemoattraction of synovial tissue macrophages but does not mention involvement of chemokine receptors at all. WO 00/46195 and WO 00/46197 concern anti-inflammatory indole derivatives which interfere with diseases mediated by the chemokines MCP-1 and RANTES. It is

35 suggested that the indole derivatives act as inhibitors of the MCP-1 receptor CCR2. WO

00/69815 suggests the use of ureido-substituted cyclic amine derivatives as inhibitors of the chemokines MCP-1 and MIP-1 $\alpha$  involved in a variety of diseases.

Chemokines and their receptors are not only found in the peripheral immune system. It has become clear recently that chemokines are also expressed in brain during development and brain pathology (for review see also: 6, 7, 8, 9). One of the first described and most prominent chemokines in brain is monocyte chemoattractant protein-1 (MCP), which is found in brain tissue after ischemia (10, 11), Alzheimer's disease (12), and Multiple sclerosis (13, 14 15). Within the damaged brain MCP-1 is produced by both astrocytes and microglia (10) and mediates, presumably, the infiltration by monocytes/macrophages and lymphocytes (16, 17). Both astrocytes and microglia are not only capable to produce chemokines. They also are involved in chemokine signaling since it is known that glial cells itself express functional chemokine receptors (18, 19, 20). In cultured microglia and astrocytes MCP-1 induces transient increases in intracellular Ca<sup>2+</sup> and/or chemotaxis (20, 21, 22, 23, 24). Although cultured glial cells (astrocytes and microglia) respond to MCP-1 stimulation, possible mRNA expression of the corresponding chemokine receptor for MCP-1 (CCR 2) (25, 26) is controversial in glial cells (18, 24, 9), and so far CCR2 mRNA expression has not been found in astrocytes.

The invention provides the insight that, possibly instead of activating the CCR-2 receptor, in brain cells such as glial cells MCP-1 activates at least one other CC chemokine receptor, a receptor earlier known as orphan receptor L-CCR in the mouse or CRAM-B in humans, which we from now on will address as CCR12 or CCR11, depending on final classification by the committee on nomenclature, see also figure 8.

With this insight, the invention provides a method for obtaining or identifying an agonist or antagonist of degenerative of inflammatory disease comprising testing a candidate agonist or antagonist compound in a method according to one as provided herein and determining said compound's capacity to modulate or mimic MCP-1 binding to said receptor in said method. After synthesizing the thus identified compound in desirable quantities, the invention thus allows the production of an agonist or antagonist of degenerative of inflammatory disease and it is use in the preparation of a pharmaceutical composition for the treatment said disease, and it provides a method of treatment of a subject prone to or having such a disease comprising treating said subject with said pharmaceutical composition.

MCP-1 induced chemokine receptor activation is therefore now shown to be involved in brain pathological events such as neurodegenerative and/or neuroinflammatory disease. A new chemokine ligand-receptor pair is thus found that contributes to an endogenous inflammatory cascade in the central nervous system which is related to above identified pathological conditions.

With that insight the invention for example provides a method for identifying a candidate drug compound for the treatment of inflammatory or degenerative brain disease comprising testing said compound for its capacity to modulate MCP-1 binding with an orphan receptor commonly known as L-CCR in the mouse or CRAM-B in humans, in particular for the treatment of brain disease after ischemia, Alzheimer's disease or multiple sclerosis. The invention provides for example the characterization and the observation of mRNA expression of a novel MCP chemokine receptor CCR12 in glial cells. Evidence is here presented that astrocytes and microglia express mRNA encoding said new chemokine receptor provided here. Cloning and expressing of this new chemokine receptor revealed that MCP-1 is a chemokine ligand for this new receptor. According to the chemokine receptor nomenclature rules we suggest to designate this receptor CCR12. Since CCR12 mRNA was strongly induced by treatment with LPS both *in vitro* and *in vivo* the further insight is provided here that this receptor plays an important role in brain immunology or brain inflammatory disease.

We thus present evidence for a new MCP-1 chemokine receptor, previously, when its affinity for MCP-1 was not known, described as the orphan receptor L-CCR (27). L-CCR mRNA is expressed in mouse astrocytes and microglia and regulated by LPS both *in vitro* and *in vivo*. Since it is now found that MCP-1 is a chemokine ligand for L-CCR we designate L-CCR as CCR 12, a new chemokine receptor responsible for the well known effects of MCP-1 on glial cells. The mRNA expression of the CC chemokine receptors CCR1-5 in cultured glial cells has been, at least partially, investigated by several groups and most studies have been performed with rat and human glial cells (Table 4). Whereas, cultured astrocytes from rat and human did not show any CCR mRNA expression, expression of CCR1 mRNA was found in mouse astrocytes (19). In rat and human microglia mRNA expression of CCR1 (19, 20, 32) and CCR5 (18, 33, 20, 34, 32) has been reported. There are conflicting reports on the expression of CCR2 and CCR3 mRNA in cultured microglia cells. Low CCR2 mRNA expression was found in cultured microglia by Boddeke et al. (20) and McManus et al., (32), whereas no CCR2 mRNA in cultured microglia was found by others (18, 24). CCR3 mRNA expression in cultured microglia was found by He et al. (33) and McManus et al., (32) but not by Jiang

et al. (18) and Boddeke et al., (20). The three reports investigating possible expression of CCR4 mRNA in glial cells failed to detect CCR4 mRNA expression (19, 20, 32). The reasons for the opposite findings concerning expression of CCR2 and 3 mRNA are currently not clear, but might be due to species variations, different culture conditions and/or different detection techniques used (Table 4).

Since very little data are available from the literature concerning CCR mRNA expression in mouse glial cells, we investigated possible mRNA expression of CCR1 to 8 and D6 in cultured mouse microglia and astrocytes using RT-PCR. The mouse chemokine receptor D6 was included in our study since it has been described as mouse CCR9 with MCP-1 binding properties (35). However since MCP-1 signaling for D6 could not be reproduced (36) this receptor was not designated as CCR9 by the nomenclature committee and currently keeps its orphan name D6 (3). All primers used in RT-PCR experiments were positively verified using genomic mouse DNA as a template and subsequent cloning and sequencing of the PCR product. We observed mRNA expression for CCR1,3,5 and CCR1,5 in cultured microglia and astrocytes, respectively, which is in good accordance with the recent literature. No other CCR mRNA's were found.

The results clearly show, that although both microglia and astrocytes respond to MCP-1 (24; own results) cultured mouse glial cells did not express CCR2 mRNA, the receptor responding to MCP-1 or D6 mRNA, a receptor which has binding properties to MCP-1. It is thus likely that cultured mouse glial cells express another receptor for MCP-1, as was already suggested by Heesen et al., (24). RT-PCR and *in situ* hybridisation showed that both cultured astrocytes and microglia express CCR12 mRNA. The CCR12 mRNA expression in both cell types was strongly increased by stimulation with LPS. Similar results were also observed *in vivo*, where CCR12 mRNA expression in cortical glial cells was strongly induced by an intraperitoneal injection of LPS.

These results therefore clearly indicate that mouse glial cells (*in vitro* and *in vivo*) express an additional LPS regulated chemokine receptor which has not been described in glial cells before. LPS stimulated RAW 264.7 cells and CCR12 transfected HEK cells, which both express CCR12, responded in a concentration-dependent manner to MCP-1 in a chemotaxis assay indicating that MCP-1 is a CC chemokine ligand for CCR12. Except from MCP-1 several other chemokines (RANTES, MIP-1a, MIP-1b, MIP-3a, MCP-2, MCP-3, fractalkine, IP-10 and SLC) are known to be expressed in brain (6-9; own observations). Among these only RANTES, MCP-2 and MCP-3 were agonists for CCR12. The members of the MCP family MCP-1, 2 and 3 are known to activate CCR2,

but RANTES is not a chemokine ligand for CCR2, which indicates that the pharmacological profile we found for CCR12 is new and unrelated to the "ligand" profiles of other receptors (3). Taken together our data provide the insight that the effects of MCP-1 on cultured mouse glial cells described in the literature and described here are mediated via CCR12.

5 Due to multiple cloning and nomination, the nomenclature of chemokine receptors has been confusing in the past (3). In order to exclude that CCR12 encodes an already known CCR paired sequence alignment was performed. Paired sequence alignment of CCR12 with all other known mouse CC chemokine receptors (CCR1-10 and 10 D6) revealed a percentage ID between 48% and 56% on the nucleotide level, indicating that the glial CCR12 encodes a new chemokine receptor (Table 2). This assumption is corroborated by our pharmacological findings that next to members of the MCP family 15 also RANTES was able to activate CCR12. Cloning of the human analogue and its expression in HEK cells revealed that MCP-1 is a chemokine ligand for the human CCR12.

Investigating the binding of biotinylated MCP-1 and MIP-1 $\alpha$  in cultured human 20 astrocytes, it was shown that both chemokines bind to pharmacologically different receptors since binding of MCP-1 was not competitively inhibited by MIP-1 $\alpha$  and vice versa (37). CCR1-5 mRNA expression in human astrocytes, however, has not been found 25 in a recent study (32). Due to the data we provide here expression of CCR12 now offers a explanation of the pharmacological data on MCP-1 binding presented by Andjelkovic et al. (37) in human astrocytes.

Among other chemokines such as MIP-1 $\alpha$ , RANTES, IP-10, MCP-2, MCP-3 and 25 C10, MCP-1 is one of the most prominent chemokines in brain. MCP-1 is induced during most types of brain injuries including as Multiple sclerosis, Alzheimer's Disease and Stroke (10-15). Within in the brain predominately glial cells (astrocytes and microglia) are the cellular source of MCP-1 (13, 38, 10, 15). MCP-1 derived from glial cell is considered to be a factor controlling and/or initiating the infiltration of the damaged 30 brain by leukocytes (17). This assumption is corroborated by a variety of findings obtained from cultured glial cells. It was found that cultured astrocytes and microglia synthesize MCP-1 upon a variety of different stimuli including LPS, IL-1 $\beta$ , INF- $\gamma$ , TNF- $\alpha$ , TGF- $\beta$  and  $\beta$ -amyloid (39-41). Moreover, MCP-1 derived from cultured astrocytes directs the migration of leukocytes across a blood-brain barrier model (16) and the

secretion of metalloproteinases by cultured microglia was strongly induced by stimulation with MCP-1 (21).

5 Brain cells (neurons and glial cells) express various receptors for chemokines such as CCR1 and 5; CXCR2 and 4 and CX3CR (for review see: 9). The expression of chemokine receptors in all intrinsic brain cells provide the insight that chemokines contribute to an endogenous inflammatory cascade in the central nervous system which is related to pathological conditions (42). Effects of chemokines on brain cells such as 10 neuroprotection in hippocampal neurons (43), inhibition of microglial activation (44) and secretion of metalloproteinases by microglia (21) are in line with that insight. Expression of glial CCR12 mRNA *in vitro* and *in vivo* was strongly upregulated by LPS treatment, which shows that CCR12 plays an important role in the chemokine/cytokine signaling 15 cascade during brain inflammation.

The invention provides among others a method for identifying a candidate drug compound for the treatment of inflammatory or degenerative brain disease comprising 15 testing said compound for its capacity to modulate or mimic MCP-1 binding with a chemokine receptor capable of being expressed on brain glial cells, said receptor known in the mouse as L-CCR or in humans as CRAM-B and herein also named CCR-12. Such a method is for example useful for finding pharmaceutical compositions for example for the treatment of ischemia, Alzheimer's disease or multiple sclerosis. In particular, such 20 a method is useful when compounds are tested for their capacity to modulate or mimic MCP-1 binding which further comprises down-regulation of said receptor, e.g. for their antagonistic characteristics. Testing can be done *in vitro* or *in vivo*, and the invention provides cells or animals provided or transfected with a recombinant nucleic acid 25 encoding at least a functional fragment of a receptor known in the mouse as L-CCR or in humans as CRAM-B, or functional equivalent thereof, for use in such a method according to the invention.

In a preferred embodiment, testing is provided under circumstances wherein mRNA expression of said receptor is up-regulated, such as to or example mimic inflammatory conditions as can be obtained after treatment with lipopolysaccharide 30 (LPS). In the detailed description a method according to the invention is provided wherein said capacity to modulate or mimic MCP-1 binding is measured by determining chemotaxis and/or calcium signalling, however, other ways of determining receptor-ligand binding are well known in the art, and can be used as well.

It is for example provided to use said chemokine receptor capable of being 35 expressed on brain glial cells, said receptor known in the mouse as L-CCR or in humans

as CRAM-B, or functional equivalent thereof in a method according to the invention separate from cells, i.e. in a cell-free system whereby the receptor (or ligand) may be bound to a solid phase and the capacity of the candidate compound is determined by competitive assay or affinity testing.

5 Preferred is a use according to the invention wherein said receptor or functional equivalent thereof is expressed in a cultured cell (*in vitro*) to better mimic pathological conditions, especially when said cultured cell comprises a cell transfected with a nucleic acid encoding at least a functional fragment of a receptor known in the mouse as L-CCR or in humans as CRAM-B, or functional equivalent thereof, as is for example shown in  
10 detail in the detailed description for a HEK cell comprising a recombinant nucleic acid encoding at least a functional fragment of a receptor known in the mouse as L-CCR or in humans as CRAM-B, (herein also termed CCR-11 or CCR-12) or functional equivalent thereof. Alternatively, a transgenic mouse (e.g. a knock-in or a knock-out mouse for the nucleic acid in question) is provided for such use, especially when testing in a live animal  
15 or tissue therefrom is required.

The invention thus provides a method for obtaining or identifying an agonist or antagonist of neurodegenerative or neuroinflammatory disease comprising (if required synthesizing and) testing a candidate agonist or antagonist compound in a method according to any one of claims 1 to 7 and determining said compound's capacity to  
20 modulate or mimic MCP-1 binding to said receptor in said method, and provides such agonists and antagonists (if required after synthesizing the desired compound at a sufficiently large scale) for use for the preparation of a pharmaceutical composition, in particular for the treatment of neurodegenerative or neuroinflammatory disease such as  
25 Alzheimers disease, stroke, Parkinson's disease, ALS, multiple sclerosis, but use with other (chronic) inflammatory disease, such as atherosclerosis, arthritis, asthma (COPD) or rheuma is also foreseen, in particular to stop the progression of above mentioned degenerative or inflammatory diseases.

In summary we show here evidence for the expression of a new LPS regulated chemokine receptor in glial cells *in vitro* and *in vivo*. Also, members of the MCP family  
30 and RANTES have been identified as chemokine ligands for this receptor, and we provide the insight that the known effects of MCP-1 on mouse glial cells are mediated via CCR12-induced signaling. What is more, since expression of CCR12 mRNA was highly dependent on LPS treatment we provide the insight that CCR12 participates in the chemokine signaling cascade during brain inflammation. The invention is further  
35 explained in the detailed description without limiting it thereto.

## Detailed description

### Material and methods

5

#### Chemicals

Isoflurane (Forene™) from Abbott (Baar, Switzerland). Dulbeccos modified Eagle Medium from GibcoBRL Life Technologies (Breda, Netherlands); TA vectors pCR2.1 and 10 pCRII from Invitrogen (Leek, Netherlands); digoxigenin-conjugated UTP and alkaline phosphatase conjugated sheep-anti-digoxigenin from Boehringer Mannheim (Mannheim, Germany); recombinant mouse chemokines from Pepro Tech EC Ltd (London, United Kingdom); antibodies for GFAP, ED-1 and MAC-1 from Chemicon (Temencula, USA); Fura-2 AM and all other chemicals from Sigma-Aldrich (Bornhem, Belgium).

15

#### Injection of LPS

For treatment with endotoxin, 5 week old CD-1 mice were injected intraperitoneally with LPS (50ug/25g weight) dissolved in sterile saline solution. Control animals received 20 injections with 0.9% NaCl alone. At different time points after the injection, animals were decapitated under isoflurane anaesthesia (5 animals per timepoint, 3 for RNA preparation and 2 for in-situ hybridisation) and brains were removed. Brains were lysed in GTC solution for RNA preparation and fixed with Zamboni's fixative by perfusion fixation for in-situ hybridisation experiments.

25

#### Cell cultures

##### *RAW 264.7 and HEK 293 cells*

Both RAW 264.7 and HEK 293 cells were maintained in DMEM containing 10% fetal 30 calf serum with 0,01% penicillin and 0,01% streptomycin in a humidified atmosphere (5% CO<sub>2</sub>) at 37° C.

##### *Mixed astrocyte cell cultures and cultured microglia*

Mixed astrocyte cell cultures were established as described previously (28). In brief, mouse cortex was dissected from newborn mouse pups (< 1d). Brain tissue was gently 35 dissociated by trituration in phosphate buffered saline and filtered through a cell

strainer (70 $\mu$ m  $\varnothing$ , Falcon) in DMEM. After two washing steps (200 x g for 10 min), cells were seeded in culture dishes (Nunc, 10cm  $\varnothing$ ) ( $8 \times 10^6$  cells/dish). Cultures were maintained 6 weeks in DMEM containing 10% fetal calf serum with 0,01% penicillin and 0,01% streptomycin in a humidified atmosphere (5% CO<sub>2</sub>) at 37° C. Culture medium

5 was changed the second day after preparation and every six days thereafter. Microglia cultures were established as described previously (29). In brief, floating microglia were harvested from confluent mixed glial cultures and plated on new culture dishes. Microglia cultures were pure (> 95%) as tested by cell specific markers (ED-1 and Mac-1). For calcium measurements cells were seeded on glass coverslips. For chemotaxis 10 assays cultured microglia were left in suspension.

Reverse transcripts polymerase chain reaction (RT-PCR)

Cells and brain material were lysed in guanidinium isothiocyanate/mercaptoethanol

15 (GTC) buffer and total RNA was extracted with slight modifications according to Chomczynski and Sacchi (30).  
a) Reverse transcription: 1 $\mu$ g of total RNA was transcribed into cDNA as described (28). Potential contaminations by genomic DNA were checked for by running the reactions (35 cycles) without reverse transcriptase and using GAPDH primers in subsequent PCR 20 amplifications. Only RNA samples which showed no bands after that procedure were used for further investigation.  
b) Polymerase chain reaction: 2 $\mu$ l of the RT-reaction were used in subsequent PCR 25 amplification as described (28). See table 1 for primer sequences, cycle numbers and annealing temperature. Identification of all PCR products were checked by cloning into PCR2.1 (Invitrogen) and subsequent sequencing.

Cloning and expression of CCR12 in HEK cells

Primers to amplify the full length sequence for mouse CCR12 have been chosen

according the sequence for L-CCR (Accession number: AB009384). The full length mouse

30 CCR12 coding sequence was amplified from cDNA derived from LPS stimulated microglia with the following primers: forward, 5'-TATCAAGCAACCTGCCTCAA; backward 5'-TGGCATAAAACAATGTGAAGAGA.

Sequence similarity searches using the mouse CCR12 sequence and human databases gave high homology of mouse CCR12 with the human orphan chemokine receptor

CRAM-B (Accession number: AF015525). The following primers were designed to get the full length sequence for the human CCR12. Forward, 5'-CCCAGTGGGCAGTCTGAA; backward, 5'-CTTGCATTTGGTGGATGCTA.

The resulting PCR products were cloned in PCR2.1 (Invitrogen) for sequencing and 5 subcloned into the *Bam*H I-*Not* I sites of pcDNA 3.1 (Invitrogen) for transfection. 1ug of the plasmid was transfected with 6ul Fugene (Roche Molecular Biochemicals) in HEK 293 cells according to the manufacturer's instructions. Stably transfected cells were selected with G418 500ug/ml for approx. 2 weeks and the resulting cell clones were checked by RT-PCR for CCR12 mRNA expression.

10

Alignment of mouse CCR12 with other CCR's

Paired alignment of the mouse CCR12 with other CCR's was performed using the alignment tool ClustalW at European Bioinformatics Institute (EBI), homepage <http://www.ebi.ac.uk>.

15

#### Determination of intracellular calcium

For calcium measurements, cells were cultured on poly-L-lysine coated glass coverslips. In order to load the cells with Fura-2 AM the cells were incubated for 30 min at 37°C in loading buffer containing: (in mM) NaCl 120, HEPES 5, KCl 6, CaCl<sub>2</sub> 2, MgCl<sub>2</sub> 1, glucose 5, NaHCO<sub>3</sub> 22, Fura-2 AM 0.005; pH 7.4. The coverslips were fixed in a perfusion chamber (37° C) and mounted on an inverted microscope. Fluorimetric measurements were done using a sensicam CCD camera supported by Axolab<sup>®</sup> 2.1 imaging software. Digital images of the cells were obtained at an emission wavelength of 510 nm using paired exposures to 340 and 380 nm excitation wavelength sampled at a frequency of 1 Hz. Fluorescence values representing spatial averages from a defined pixel area were recorded on-line. Increases in intracellular calcium concentrations were expressed as the 340/380 ratio of the emission wavelengths. Compounds were administered using a pipette positioned at a distance of 100-300  $\mu$ m from the cells.

30

#### Chemotaxis assay

Cell migration in response to chemokines was assessed using a 48-well chemotaxis microchamber (NeuroProbe). Chemokine stock solutions were prepared in PBS and further diluted in medium for use in the assay. Culture medium without chemokines served as a control in the assay. 27ul of the chemoattractant solution or control medium were added to the lower wells, lower and upper well were separated by a

polyvinylpyrrolidone-free polycarbonate filter (8  $\mu$ m pore size) and 50000 cells per 50 $\mu$ l were used in the assay. Determinations were done in hexuplicate. The chamber was incubated at 37°C, 5% CO<sub>2</sub> in a humidified atmosphere for 120 min. At the end of incubation the filter was washed, fixed in methanol and stained with toluidine blue.

5 Migrated cells were counted with a scored eyepiece (3 fields (1mm<sup>2</sup>) per well) and migrated cells per chamber were calculated. The data are presented as mean values  $\pm$  S.D. and were analysed by Students *t*-test. P values  $\leq$  0.01 were considered significant.

10 Immunohistochemistry and *in-situ* hybridisation

Immunochemistry and *in-situ* hybridisation was carried out as described (31). In brief, prior to immunohistochemical processing and between the incubation steps, the sections were washed in 0.9% saline dissolved in 0.05 M Tris, pH 7.4 (TBS). All antisera were diluted in TBS containing 0.3% Triton X-100, 1% bovine serum albumin (BSA) and heparin (5mg/ml). Sections were preincubated in 5% BSA in TBS for 30 min and incubated overnight with GFAP and ED-1. Antibody-antigen reactions were detected using the biotin-streptavidin method and the complex was visualised with diaminobenzidine (DAB)/H<sub>2</sub>O<sub>2</sub>. In case of fluorescence detection FITC conjugated streptavidin was used to visualise the antibody-antigen complex. For *in-situ* hybridisation CCR12 PCR product was cloned into the dual promoter PCR II vector and linearised. CCR12 sense and antisense probes were synthesised by run off transcription and the use of digoxigenin-conjugated UTP according to the manufacturers protocol (Boehringer Mannheim). Slides were rinsed in PBS and digested with 10  $\mu$ g/ml proteinase K for 0.5 h at 37 °C. Subsequently, sections were rinsed in 2x SSC (1 x SSC: 25 150 mM NaCl, 15 mM Na citrate), dehydrated in an ethanol series and dried.

Sections were hybridised overnight at 60 °C in a solution containing 50% formamide, 0.3 M NaCl, 10 mM Tris (pH 8.0), 1 mM EDTA, 0.05% tRNA, 1 x Denhardt's solution and 10% dextran sulfate. Final probe concentrations in hybridisation buffer were 1-5 ng/ $\mu$ l. After hybridisation, 30 the sections were treated with 10  $\mu$ g/ml of ribonuclease A for 0.5 h at 37 °C and washed in 0.1x SSC at 65 °C.

The immunological detection of the digoxigenin labeled RNA-RNA complex was preceded by a 0.5 h pre-incubation at room temperature in 0.1 M Tris, 0.15 M NaCl, pH 7.5 (buffer 1), containing 5% BSA. Slides were incubated for 2h at room temperature with

the alkaline phosphatase conjugated sheep-anti-digoxigenin, diluted 1:500 in buffer 1, containing 2% BSA. After thorough rinsing in buffer 1 and a 10 min pre-incubation in an alkaline buffer solution (ABS: 0.1 M Tris, 0.1 M NaCl, 0.05 M MgCl<sub>2</sub>.6H<sub>2</sub>O, pH 9.5), the alkaline phosphatase was revealed with a freshly prepared solution of 0.34 mg/ml nitroblue tetrazoleum and 0.17 mg/ml 5-bromo-4-chloro-3 indolyl phosphate in ABS. Endogenous non-intestinal phosphatase activity was inhibited by the addition of levamisole (0.24 mg/ml) to the staining solution. The color development was done overnight and terminated by placing the slides in a buffer solution, consisting of 0.01 M Tris, 1 mM EDTA, pH 8.5. The dark purple precipitate indicating the presence of hybridised mRNA was revealed with bright-field microscopy. Control experiments included hybridisation with digoxigenin-labeled sense probes and hybridisation after treatment of the sections with RNase.

## Results

15

### *Expression of CCR mRNA's in RAW cells*

The expression of an orphan LPS inducible chemokine receptor (L-CCR) in the mouse macrophage cell line RAW 264.7 was previously described by Shimada et al. (27). Later, we came to designate L-CCR as CCR12, and we use this term hereinafter for the sake of convenience. In order to investigate possible mRNA expression of this receptor in other cell types, we designed primers for RT-PCR experiments and validated the primers using cDNA derived from RAW 264.7 cells. Results similar to those described by Shimada et al., (1998) were found; mRNA for CCR12 was strongly upregulated in RAW cells by stimulation with LPS (100ng/2h) (Fig. 1). Using RT-PCR analysis (35 cycles) no other mRNA encoding mouse CCR's (CCR1-8 and D6) was detected in cDNA derived from control or LPS stimulated RAW 264.7 cells. This indicates that CCR12 is the only  $\beta$ -chemokine receptor in these cells. Genomic mouse DNA served as a positive control for the primers (CCR1-8 and D6) used.

30

### *Expression of CCR mRNA in cultured mouse astrocytes and microglia*

In cultured mouse microglia mRNA for CCR1, 3 and 5 was detected (Fig 2A). No mRNA for CCR's 2,4,6,7,8 and D6 was found in these cells (35 cycles RT-PCR) (data not shown). Untreated microglia did show basal expression levels for CCR12 mRNA and this expression was upregulated by a 2h stimulation with 100ng/ml LPS (Fig. 2B). Similar but less pronounced effects were found after 2h stimulation with 10 and 1 ng/ml LPS

(data not shown). LPS induction of CCR12 mRNA in cultured microglia peaked at 2h and declined to baseline expression after 8h (data not shown).

Using RT-PCR, mRNA expression for CCR1 and 5 was detected in cultured astrocytes (Fig 2C). No other CCR mRNA (2,3,4,6,7,8 and D6) was found in these cells (35 cycles

5 RT-PCR) (data not shown). Similar to microglia, untreated cultured astrocytes showed basal mRNA expression for CCR12, which also was upregulated after a 2h stimulation with LPS (100ng/ml) (Fig 2D). Treatment with 1 and 10 ng/ml LPS had a similar but less pronounced effect (data not shown). In cultured astrocytes a comparable time dependency for the LPS effects was detected as it was found for cultured microglia (data 10 not shown). No CCR12 mRNA expression was detected in cDNA derived from cultured cortical neurons (data not shown).

In order to verify the results obtained with RT-PCR *in situ* hybridisation was combined with immune histochemistry. Mixed glial cultures were stimulated for 2h with LPS (100 ng/ml) and stained with ED-1 and GFAP to detect microglia and astrocytes respectively.

15 ED-1 positive microglia (brown reaction product) showed a positive signal for CCR12 *in situ* hybridisation (purple reaction product) (Fig 3A). Note that an *in situ* signal is also visible in ED-1 negative cells, which might be in this case an astrocyte (arrowhead) (Fig. 3A). CCR12 positive astrocytes (purple reaction product) (arrows) became visible by staining with GFAP (brown reaction product) (Fig 3B). Note that also GFAP negative 20 cells are *in situ* positive, which is this case most likely a microglia cell (arrowhead) (Fig 3B).

#### *Expression of CCR12 mRNA in brain tissue*

Mice were injected intraperitoneally with LPS (50ug/25g weight) or with 0.9% NaCl and

25 brains were removed after 2, 4, 8, 12 and 24h for RT-PCR analysis or *in situ* hybridisation. Injection with control NaCl solution did not affect expression of CCR12 mRNA in brain tissue (data not shown). In contrast, injection of LPS induced the expression of CCR11 mRNA 2, 4 and 8h after the injection. 12h after the injection of LPS CCR12 mRNA expression returned to baseline levels (Fig 4). These results were 30 verified by *in situ* hybridisation experiments. No CCR12 mRNA positive cells were found in control brains (Fig 5A), 2h after injection of LPS many CCR12 positive cells were observed in the cortex of the LPS treated mice (Fig 5B), 24h after the injection CCR12 *in situ* hybridisation signal returned to control levels (Fig 5C). Combinations of *in situ* hybridisation (purple reaction product) (Fig 5D) and immuno-histochemistry (GFAP 35 fluorescence) (Fig 5E) revealed that GFAP positive astrocytes express CCR12 mRNA in

mouse cortex (see Fig 5F for overlay of 5D and E). For technical reasons it was not possible to co-localize CCR12 mRNA with microglial markers *in vivo*. Since CCR12 mRNA positive and GFAP negative cells were found in brain it is suggested that there are celltypes different from astrocytes expressing CCR12 mRNA, which might be microglia

5 as observed in cell culture studies.

*Effect of MCP-1 and RANTES (CCL5) on chemotaxis and calcium signaling of RAW 264.7 cells*

In order to find possible chemokine ligands for CCR12 chemotactic activity and 10 mobilisation of intracellular calcium was determined in LPS treated RAW cells. MCP-1 induced concentration-dependent chemotaxis of RAW cells with an EC50 value of approximately 0.1 nM (Fig 6A). Similar results were obtained using RANTES, which was less potent with an EC50 value of approximately 1nM (Fig 6A). The CC chemokine MIP-1 $\alpha$  (CCL3) did not induce chemotaxis in RAW cells (data not shown). Both 15 chemokines RANTES and MCP-1 were also found to induce intracellular calcium transients in RAW cells (Fig 6B).

*Chemotaxis of CCR12 transfected HEK cells*

In order to further investigate its agonist responsiveness we cloned mouse CCR12 from 20 LPS treated microglia and subsequently the receptor was expressed in HEK 293 cells. Sequencing of the glial CCR12 revealed 99% identity with the sequence previously published for the orphan receptor (27). Mock transfected HEK cells did not migrate towards a chemotactic gradient of MCP-1, whereas CCR12 transfected HEK cells concentration dependently migrated in response to MCP-1 (Fig 7A). Moreover, MCP-1 25 induced intracellular calcium transients in CCR12 transfected HEK cells (Fig. 7B). Among several other chemokines found in brain (RANTES, Fractalkine (CX3CL1), MIP-1 $\alpha$ , MIP-1 $\beta$  (CCL4), MIP-3 $\alpha$  (CCL20), IP-10 (CXCL10), MCP-2 (CCL8), MCP3 (CCL7) and SLC (CCL21)) only RANTES, MCP-2 and MCP-3 were found to induce chemotaxis of CCR12 transfected HEK cells (Tab. 3). In set of preliminary experiments we 30 performed chemotaxis assays with HEK cells expressing the human CCR12 and verified that MCP-1 is a chemokine ligand also for human CCR12. (data not shown).

*Effect of MCP-1 on calcium and chemotaxis of cultured microglia*

Stimulation of chemotaxis of cultured mouse astrocytes by MCP-1 has already been

5 shown by Heesen et al., (1996). Effects of MCP-1 on cultured microglia were only shown so far for rat microglia (20, 22) and fetal human microglia (23) but not for mouse microglia. We therefore determined the effects of MCP-1 on intracellular calcium transients and chemotaxis of cultured mouse microglia. Similar to microglia from other species, 10 nM MCP-1 induced chemotaxis of cultured mouse microglia; migration of  
10 untreated cells,  $29 \pm 13$  (cells/mm<sup>2</sup>), migration of cells stimulated with 10 nM MCP-1  $170 \pm 42$  (cells/mm<sup>2</sup>) (n=4). Chemotaxis was determined as described in materials and methods. Moreover intracellular calcium transients in cultured microglia were observed upon stimulation with MCP-1 (data not shown).

Table 1

Primer sequences for mouse CCR's

Gene	Primer sequences (5`-3`)	PCR product (bp)
CCR1	GTGGTGGGCAATGTCCTAGT TCAGATTGTAGGGGGTCCAG	658
CCR2	GTATCCAAGAGCTTGATGAAGG G GTGTAATGGTGATCATCTTGT GGA	532
CCR3	GCACCACCCGTGAAAAAGT CGAGGACTGCAGGAAACTC	521
CCR4	AGGCAAGGACCCGTGACCTAT GGACTCGCTGTAAGAGGAGC	644
CCR5	ATTCTCCACACCCGTTCG TCAGGCTTGCTTGCTGGAA	350
CCR6	GTGGTGTGACCTTGCCTT AGGAGGACCATGTTGTGAGG	656
CCR7	AACGGGCTGGTGATACTGAC ATGAAGACTACCACACGGC	596
CCR8	TTCTGCCTCGATGGATTAC GCTTCCACCTCAAAGACTGC	591
D6	TCTTCATCACCTGCATGAGC TATGGAAACCACAGCATGAA	400
CCR12	CTGGGGTGTATCTTGGT AACCAGCAGAGGAAAGCAA	489
GAPDH	CATCCTGCACCACCAACTGCTTA G GCCTGCTTCACCACCTCTTGT G	346

Table 2

Comparison of CCR12 with all other cloned mouse CCR's by nucleic acid sequence

5 alignment

Beta chemokine receptor (mouse)	Accession Number	Percentage ID after alignment with glial CCR12
CCR1	U28404	52.8
CCR2	U51717	49.6
CCR3	U29677	54.3
CCR4	X90862	51.5
CCR5	U83327	53.1
CCR6	AB009369	51.6
CCR7	L31580	50.2
CCR8	Z98206	56.6
CCR9	AJ132336	49.1
CCR10	AF215982/ AF215983	48.0
D6	Y12879	50.0

10

Table 3

Effect of various chemokines on chemotaxis of CCR12 transfected HEK 293 cells

Chemokine (100nM)	Chemotactic effect on CCR12 transfected HEK cells
RANTES	+
MCP-1	+
MCP-2	+
MCP-3	+
MIP-1 $\alpha$	-
MIP-1 $\beta$	-
MIP-3 $\alpha$	-
Fractalkine	-
IP-10	-
SLC	-

Table 4

Expression profile of CCR mRNA in cultured glial cells from human (H), mouse (M) and rat (R).

5

	astrocytes			microglia			publication
	H	M	R	H	M	R	
CCR1	+						19
				+			20
							32
CCR2	-						24
				+			20
							18
							32
CCR3	-	-					18
				+			20
							33
							32
CCR4	-	-					19
				-			20
							32
CCR5	-	-					18
				+			20
							33
							32

### Further experiments

#### Testing in experimental allergic encephalitis (EAE) in mice

10

##### Animals

C57BL6/J mice were obtained from Jackson laboratories, and housed in groups of four, with free access to food and water. Young adult mice (8-12 weeks) were used.

15

Antigens

Recombinant myelin oligodendrocyte glycoprotein (MOG) (residues 35-55), obtained from S.Amor from the BPRC at TNO in Rijswijk, was used as antigen.

5

## Induction of EAE

Immunisation of the animals was done with 200 $\mu$ g MOG peptide, added to PBS, emulsified by sonication for 10 min at room temperature, in incomplete Freunds' adjuvans (IFA) supplemented with 60  $\mu$ g of mycobacterium. The mice were injected subcutane on day 0 and 7 at two sites on the back. In addition the mice were also given i.p. 200ng of pertussis toxin dissolved in phosphate buffered saline (PBS) intraperitoneal. The pertussis toxin was given immediately and 24 h later after immunisation with the antigen in complete Freunds' adjuvans (CFA).

10

15

Scoring in EAE

The scoring and weighing of the animals started on day 8. Grade 0 meant there were no symptoms at all. At grade one the tail was paralysed. Grade two was reached when the righting reflex was impaired. Grade three was reached when one of the hind limbs was paralysed. Grade four was given when both hind limbs were paralysed. Grade five is a 20 moribund state in which all limbs are completely paralysed. Intermediate scores of 0,5 were given. We terminated the mice at grade 3,0.

20

## Termination of the mice

25

After the termination of the mice, using Isoflurane as anaesthetic, the spinal cord and the brain were taken out and frozen in liquid nitrogen, for RNA analysis. For *in situ* hybridisation the mice were perfused with 4% paraformaldehyde (pfa) in PBS and consequently the spinal cord and brain were taken out and put in fixative. The tissue 30 was embedded in TissueTek, frozen and cut with a Reichert-Jung Frigocut 2800 microtome. Sections were caught on double-coated glasses.

35

Results

Using *in situ* hybridization assays a strong increase of the CCR11 chemokine receptor was observed during EAE. This shows that the receptor is involved in MS pathology.

5 Accordingly, the use of antagonists of CCR11 is of use in MS therapy.

Testing chronic obstructive pulmonary disease (copd) in miceMice and in vivo procedures

10 Two weeks prior to the experiment 8-10 weeks old mice C57BL/6-J were purchased from Harlan.

For sensitization (day 0), 24 mice were injected intraperitoneally with ovalbumine (OVA) 15 0.1 mg/mouse in Phoshor Buffered Saline (PBS).

For induction of allergic response (day 8), 18 mice were challenged for 5 minutes with a 2 % OVA aerosol in PBS. For control experiments, 6 mice were challenged for 5 minutes with PBS.

20 For allergen provocation (day 15), 18 mice were challenged for 20 minutes with a 1 % OVA in PBS aerosol, 6 mice were challenged for 20 minutes with PBS (control). Respectively 1, 3 and 6 hours after OVA challenge and 3 hours after PBS challenge (control) 3 mice were terminated. Both lungs of all challenged mice were isolated and 25 immediately placed in liquid Nitrogen.

On days 16, 17 and 18, mice were repetitively challenged for 20 minutes with 1% OVA (n=9) and PBS (n=3, control). On day 18, respectively 1,3 and 6 hours after OVA challenge and 3 hours after PBS challenge (control) 3 mice were terminated. Lungs were 30 isolated and immediately placed in liquid Nitrogen

All challenge protocols were performed in a specially designed perspex cage with an internal volume of 9L. A summary of the challenge protocol is given in figure 9.

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## Legends to the figures

## Figure 1

RT-PCR analysis of CCR12 mRNA expression in unstimulated (C) and LPS stimulated

5 RAW 264.7 cells. Cells were stimulated with 100ng/ml LPS for 2h. Number of cycles for GAPDH and CCR12 were 28 and 32 respectively. MM, molecular weight marker, highlighted band is 500bp. Both PCR products were run in the same gel. Similar results were found in 3 independent experiments.

## 10 Figure 2

RT-PCR analysis of chemokine receptor mRNA expression in cultured microglia (A and

B) and cultured astrocytes (C and D). Experiments were carried out as described in

materials and methods. A) CCR1, 3 and 5 mRNA was found in cultured microglia. B)

Unstimulated microglia (C) did show basal CCR12 mRNA expression which was

15 upregulated by a 2h stimulation with 100ng/ml LPS. C) In cultured astrocytes mRNA expression for CCR1 and 5 was found. D) Control astrocytes (D) did show basal expression of CCR12 mRNA which was upregulated by a 2h stimulation with 100ng/ml LPS. Number of cycles for GAPDH and CCR12 were 28 and 32 respectively. MM, molecular weight marker, highlighted band is 500bp. For B and D were both PCR

20 products run in the same gel. Similar results were found in 3 independent experiments.

## Figure 3

In situ hybridisation in combination with immuno histochemistry shows CCR12 mRNA

25 expression in LPS stimulated cultured microglia and astrocytes. Cultured glial cells were

stimulated for 2h with LPS (100ng/ml) and fixed as described in material and methods.

A) Cells were incubated with ED-1 antibody to stain microglia (brown reaction product).

The combination with in situ hybridisation (purple reaction product) revealed that ED-1 positive microglia also express CCR12 mRNA (arrows). The ED-1 negative but CCR12 mRNA positive cell might be an astrocyte (arrowhead). B) Cells were incubated with

30 GFAP antibody to stain astrocytes (brown reaction product). The combination with in situ hybridisation (purple reaction product) clearly showed that GFAP positive cells also express CCR12 mRNA (arrows). The GFAP negative but CCR12 mRNA positive cell is most likely a microglia (arrowhead). Bar 10um.

## Figure 4

Effect of LPS injection on CCR12 mRNA expression in mouse brain. RT-PCR experiments revealed that CCR12 mRNA expression in mouse brain was induced 2, 4 and 8h after the injection of LPS (50ug/25g weight). 12h after the injection CCR12 mRNA expression returned to control levels. Number of cycles for GAPDH and CCR12 were 28 and 32 respectively. MM, molecular weight marker, highlighted band is 500bp. Both PCR products were run in the same gel. Similar results were found in 3 independent experiments.

## Figure 5

CCR12 mRNA in situ hybridisation in the cortex of LPS injected mice and identification of astrocytes as CCR12 mRNA expressing cells. A) Lack of CCR12 mRNA expression in control brain, only unspecific staining is visible. B) 2h after the injection of LPS CCR12 mRNA expression is induced in many cells. C) CCR12 mRNA returned to control levels 24h after the injection of LPS. D) CCR12 mRNA positive cells in higher magnification in mouse brain 2h after LPS injection. E) Fluorescence micrograph of the same region as in D stained with anti-GFAP to detect astrocytes. F) Electronic overlay of D and E to verify that some CCR12 positive cells stain for GFAP indicating that astrocytes are a cellular source of CCR12 mRNA. Note that there are also CCR12 mRNA positive and GFAP negative cells indicating that at least one other cell type different from astrocytes express CCR12 mRNA. Bar in A-C 50um; in D-F 10um.

## Figure 6

Effects of MCP-1 and RANTES on chemotaxis and intracellular calcium transients of cultured RAW 264.7 cells. A) Concentration-dependent chemotaxis of cultured RAW cells induced by MCP-1 and RANTES. The graphs show the results of a typical chemotaxis experiment performed in hexuplicate for each concentration of MCP-1 and RANTES. Data are means  $\pm$  SEM (n=4); similar results were obtained in 4 independent experiments. B) Figure shows a typical example of an induction of intracellular calcium transients in RAW cells by MCP-1 or RANTES, arrow indicates the timepoint of stimulation.

## Figure 7

Effect of MCP-1 on chemotaxis and intracellular calcium transients of CCR12 transfected HEK cells. A) Chemotaxis of MOK-transfected HEK cells was not affected by

MCP-1, whereas CCR12 transfected HEK cells migrated concentration dependend when stimulated with MCP-1. The graphs show the results of a typical chemotaxis experiment performed in hexuplicate for each concentration of MCP-1. Data are means  $\pm$  SEM; similar results were obtained in 3 independent experiments. B) Typical example of a 5 MCP-1 (100nM) induced intracellular calcium transient in CCR12 transfected HEK cells.

Figure 8

Multiple nucleotide sequence alignment of human (hCCR12) or mouse (mCCR12) 10 chemokine receptor sequences herein addressed as CCR12 or CCR11.

Figure 9

Challenge protocol used in the COPD experiment

15

Figure 10

mRNA expression in the COPD experiment after 1 allergen provocation with 1% OVA

20 Figure 11

mRNA expression in the COPD experiment after 4 days of repetitive allergen provocation with 1% OVA

25

## Claims

1. A method for identifying a candidate drug compound for the treatment of inflammatory or degenerative brain disease comprising testing said compound for its capacity to modulate or mimic MCP-1 binding with a chemokine receptor capable of being expressed on brain glial cells, said receptor known in the mouse as L-CCR or in humans as CRAM-B.

5 2. A method according to claim 1 wherein said disease comprises ischemia, Alzheimer's disease or multiple sclerosis.

10 3. A method according to claim 1 or 2 wherein said capacity to modulate or mimic MCP-1 binding further comprises down-regulation of said receptor.

15 4. A method according to claim 3 wherein said capacity is tested *in vitro*.

5 5. A method according to claim 4 wherein mRNA expression of said receptor is up-regulated.

20 6. A method according to claim 5 wherein said expression is upregulated by treatment with lipopolysaccharide (LPS).

7. A method according to anyone of claims 1 to 6 wherein said capacity to modulate or mimic MCP-1 binding is measured by determining chemotaxis.

25 8. Use of a chemokine receptor capable of being expressed on brain glial cells, said receptor known in the mouse as L-CCR or in humans as CRAM-B, or functional equivalent thereof in a method according to any one of claims 1 to 7.

30 9. Use according to claim 8 wherein said receptor or functional equivalent thereof is expressed in a cultured cell.

35 10. Use according to claim 9 wherein said cultured cell comprises a cell transfected with a nucleic acid encoding at least a functional fragment of a receptor known in the mouse as L-CCR or in humans as CRAM-B, or functional equivalent thereof.

11. Use according to claim 10 wherein said cell comprises a HEK cell.
12. A cell comprising a recombinant nucleic acid encoding at least a functional fragment of a receptor known in the mouse as L-CCR or in humans as CRAM-B, or functional equivalent thereof.
13. An animal comprising a cell according to claim 12.
- 10 14. A method for obtaining or identifying an agonist or antagonist of degenerative of inflammatory disease comprising testing a candidate agonist or antagonist compound in a method according to any one of claims 1 to 7 and determining said compound's capacity to modulate or mimic MCP-1 binding to said receptor in said method.
- 15 15. An agonist or antagonist of degenerative of inflammatory disease obtainable or identifiable by a method according to claim 14.
16. Use of an agonist or antagonist according to claim 15 for the preparation of a pharmaceutical composition.
- 20 17. Use of claim 14 for the preparation of a pharmaceutical composition for the treatment of neurodegenerative or neuroinflammatory disease.
- 25 18. A pharmaceutical composition comprising an agonist or antagonist according to claim 15.
19. A method for the treatment of a neurodegenerative or neuroinflammatory disease comprising treating an individual with a pharmaceutical composition according to claim 18.

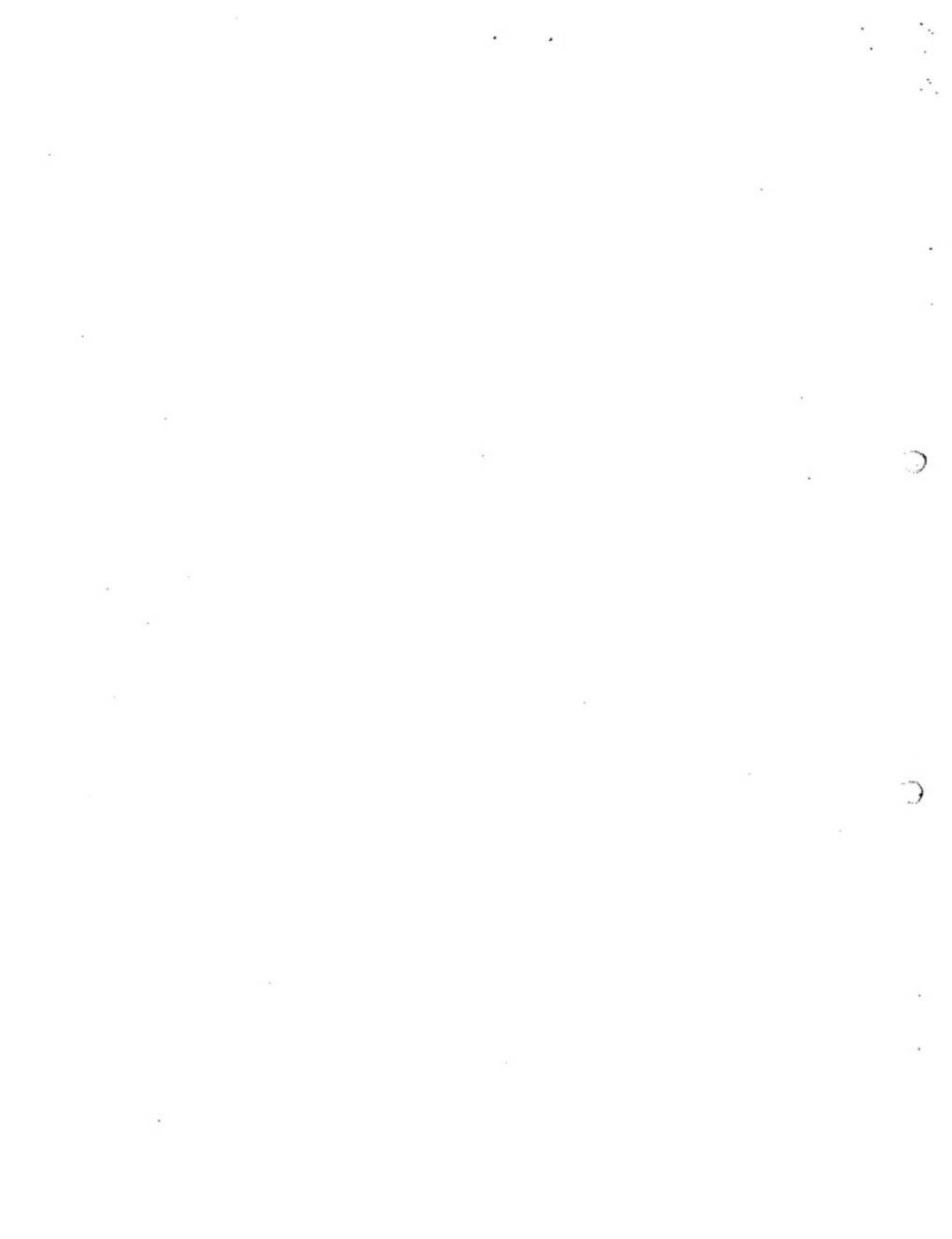


Fig. 1



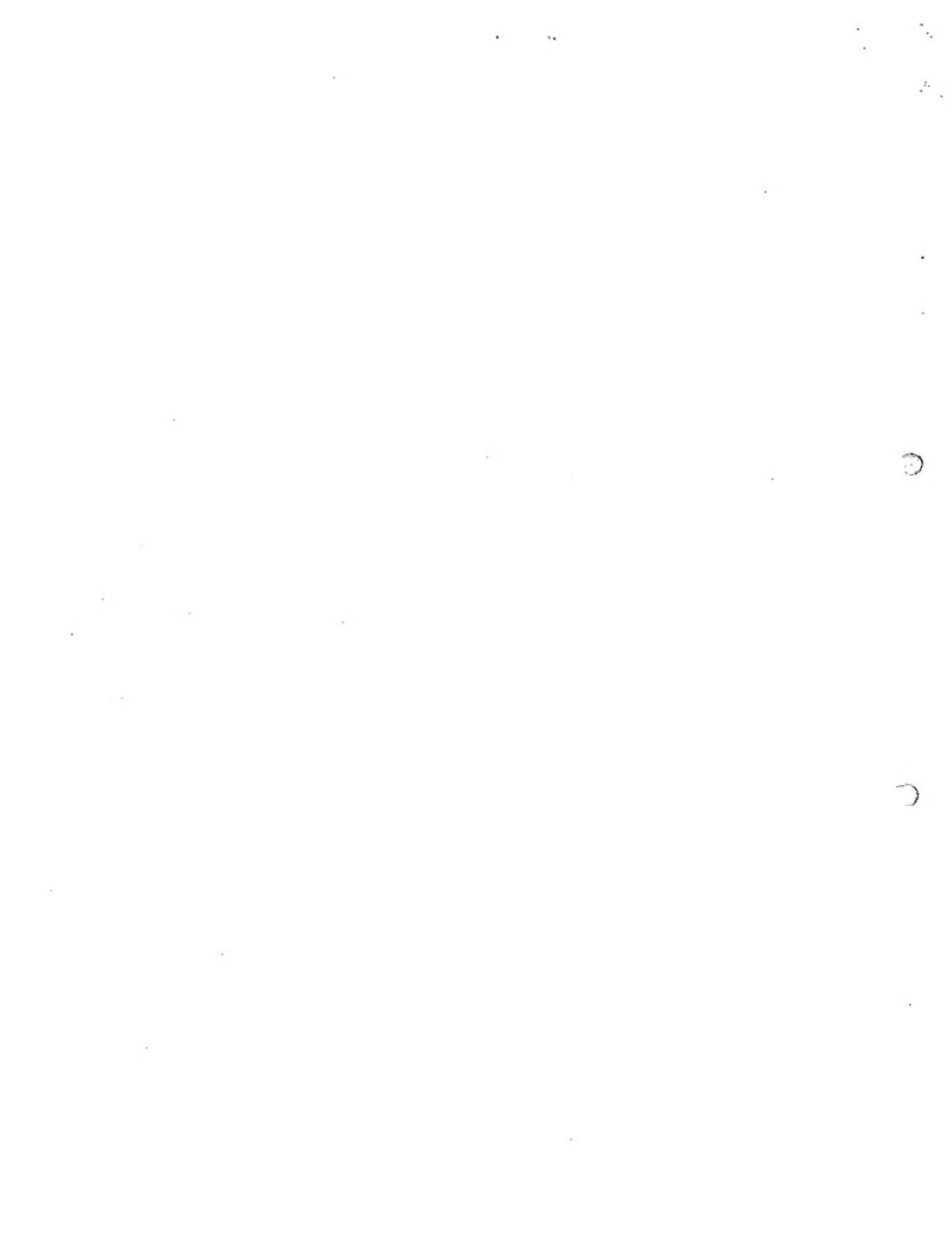
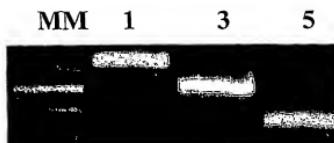
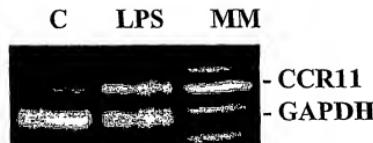


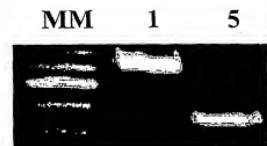
Fig. 2



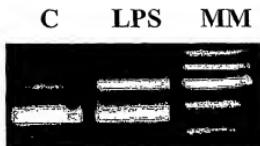
A



B



C



D

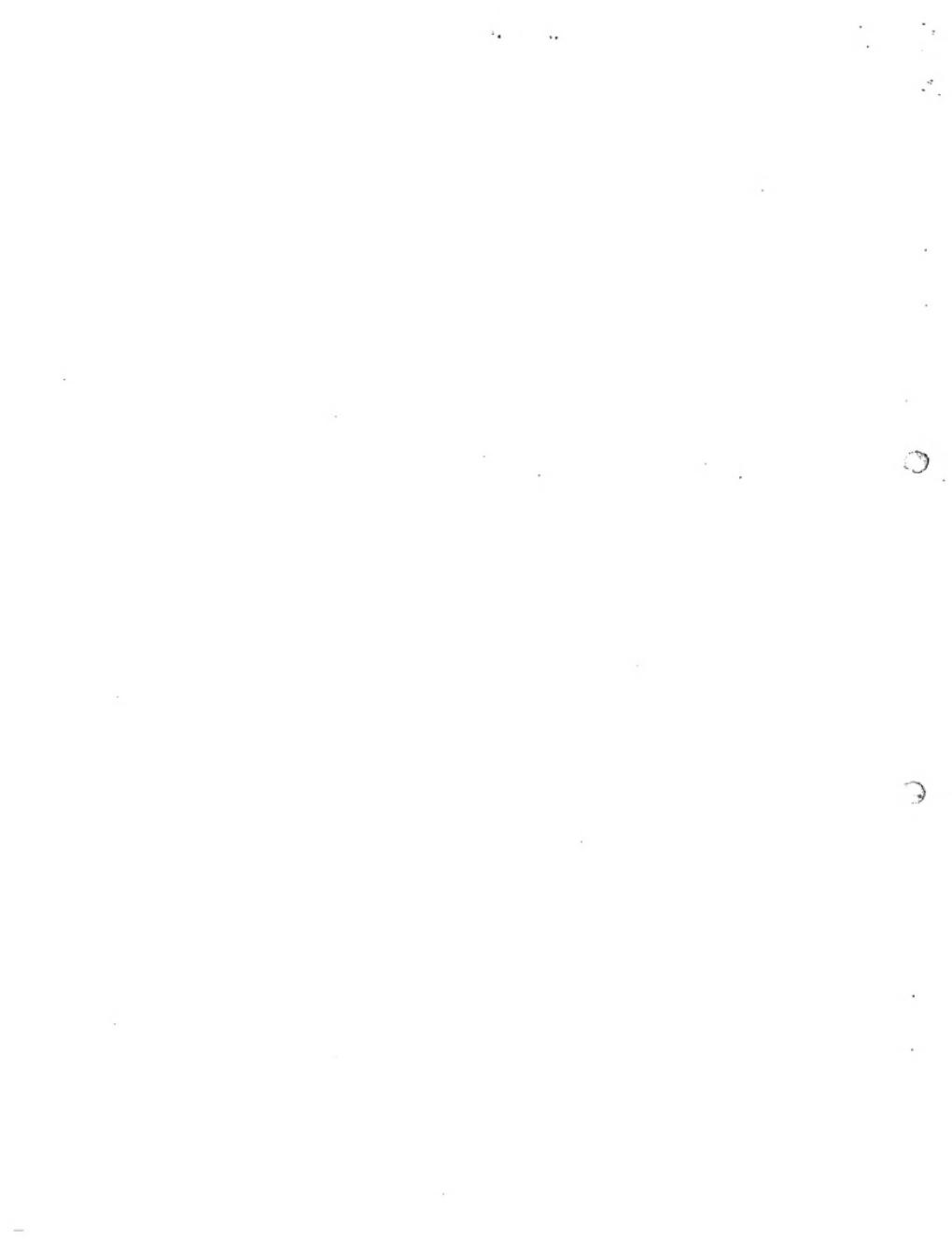


Fig. 3



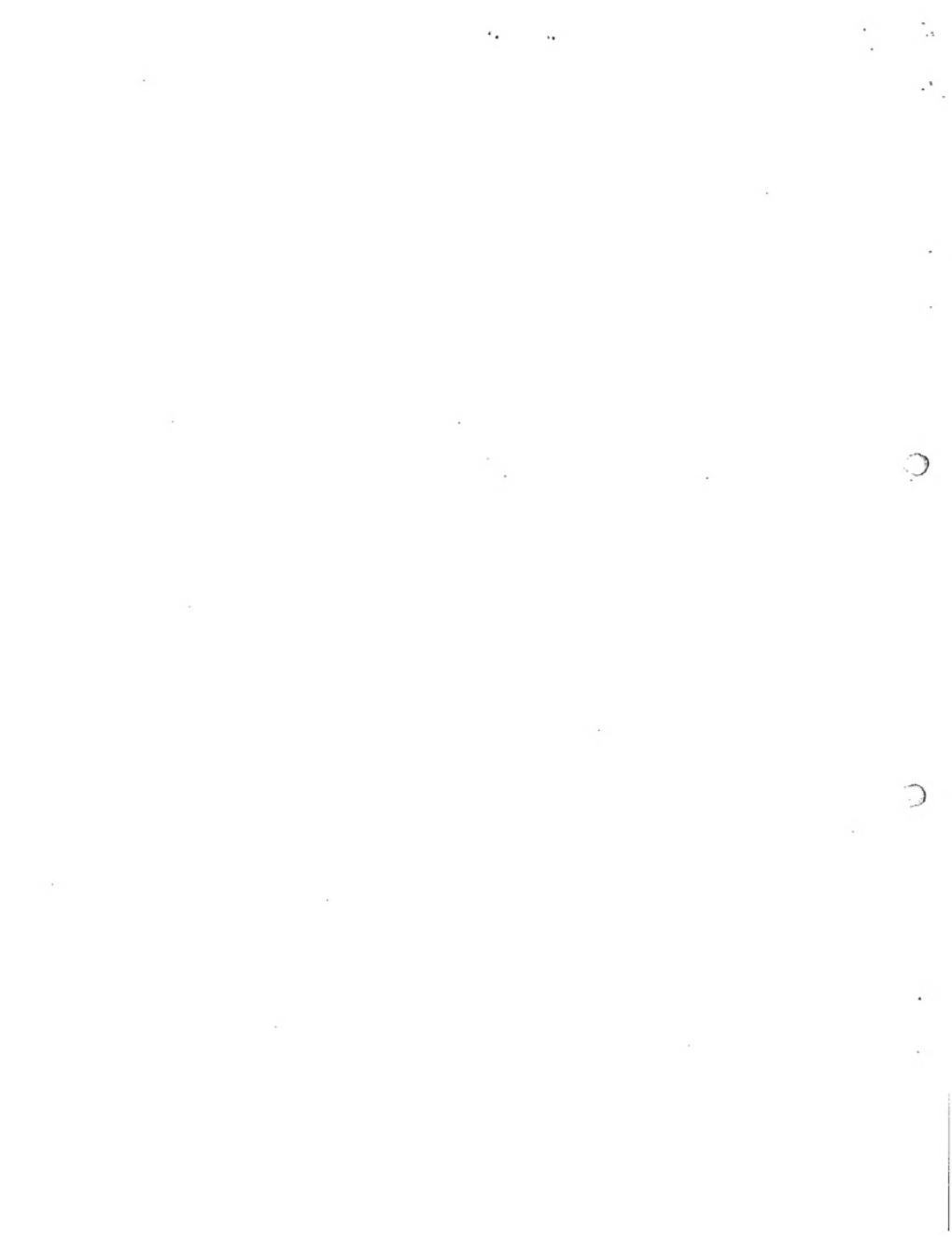
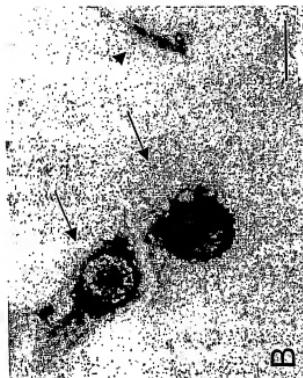


Fig. 4



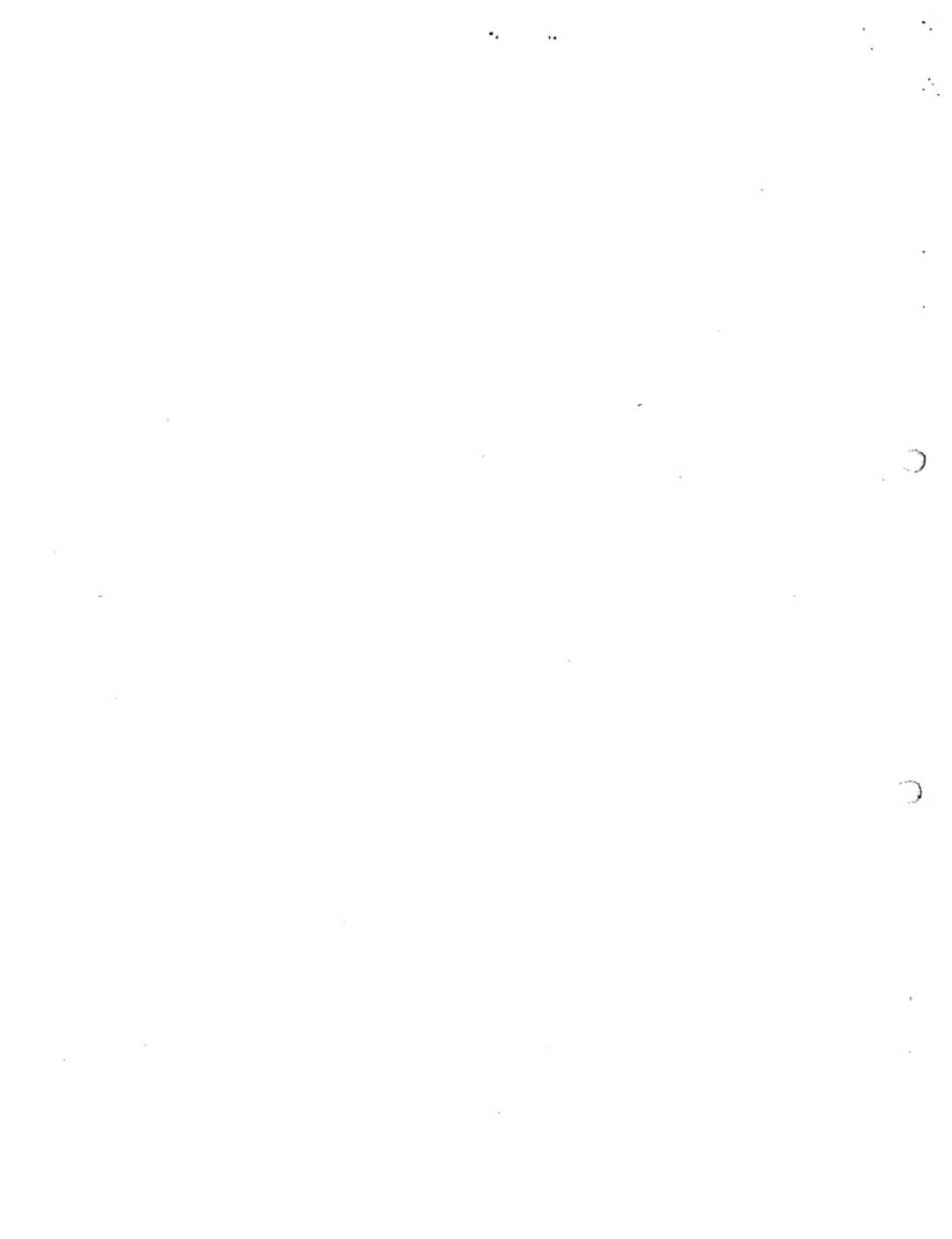
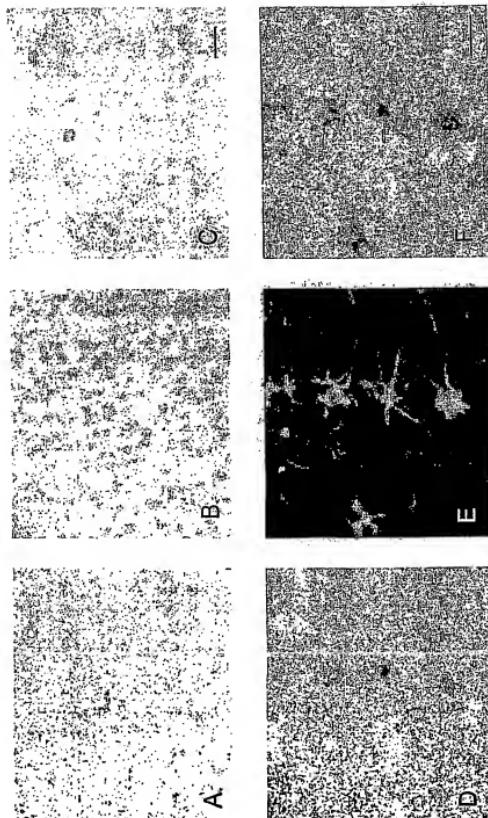
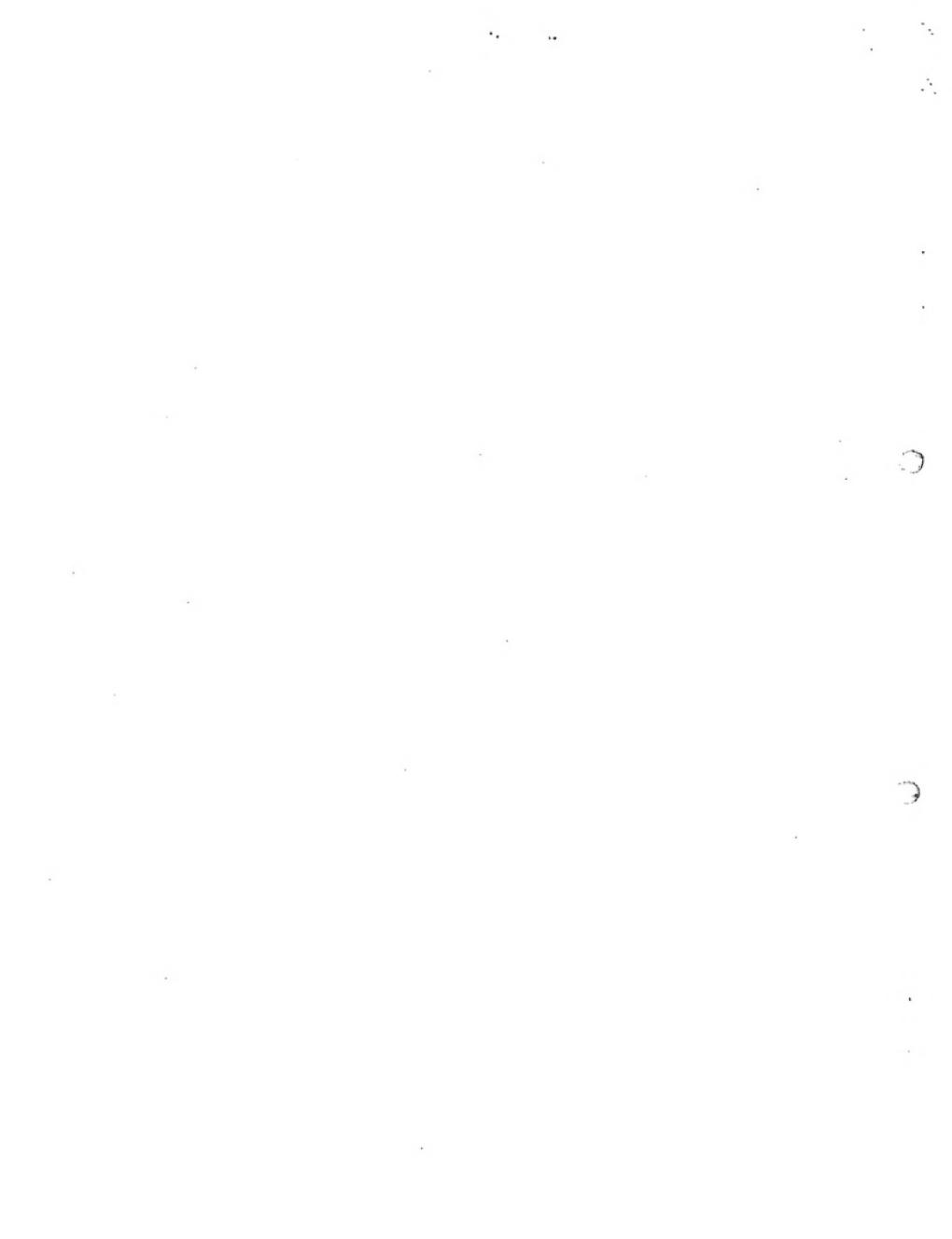


Fig. 5

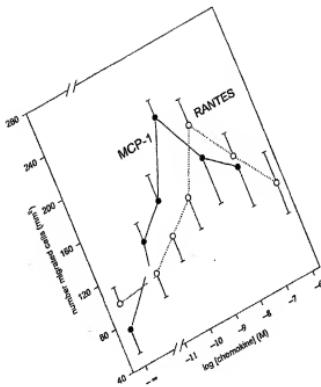




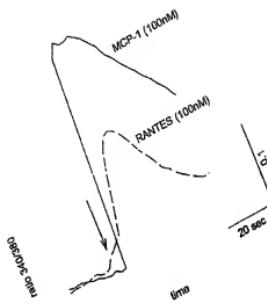
WO 02/057779

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Fig. 6



A



B

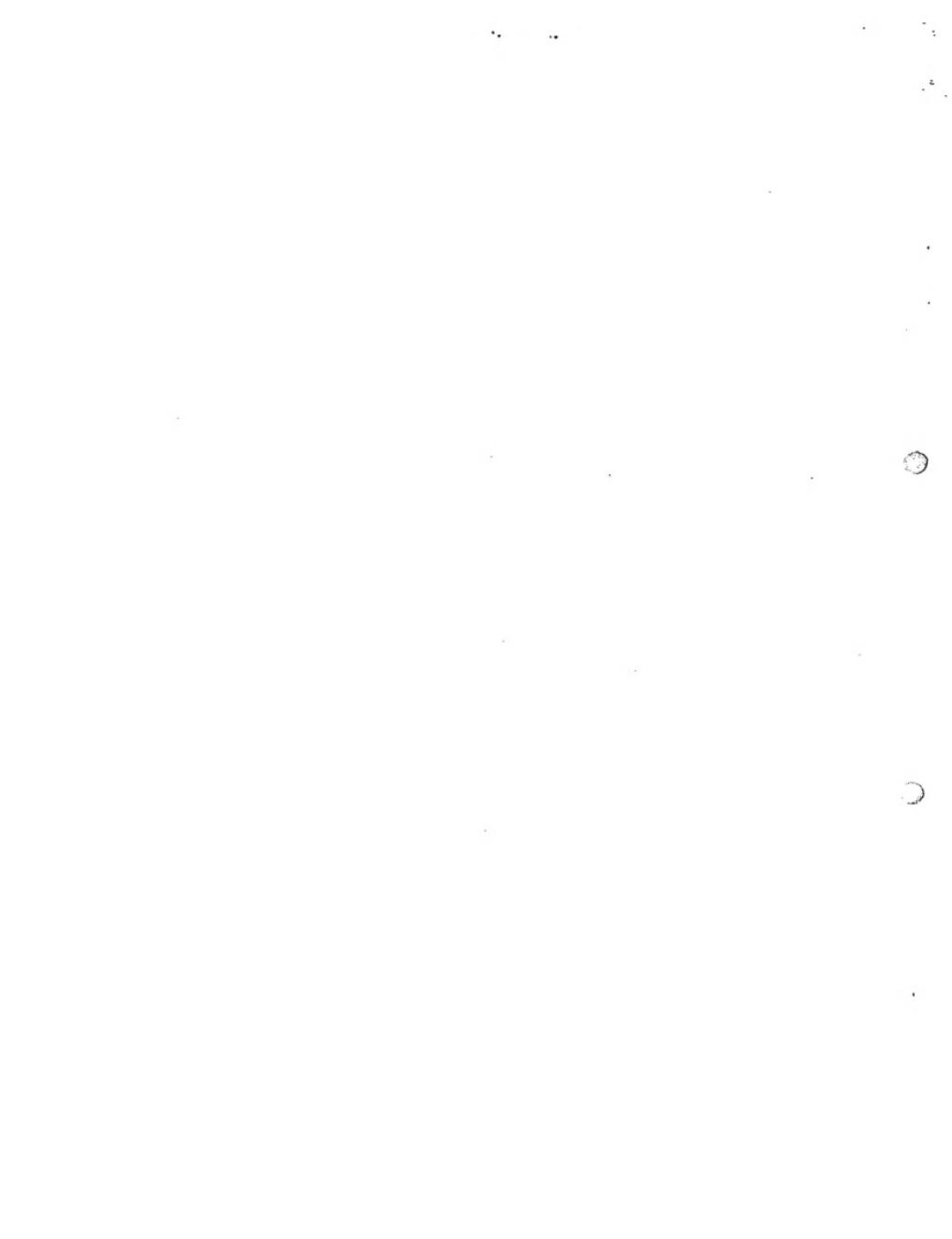
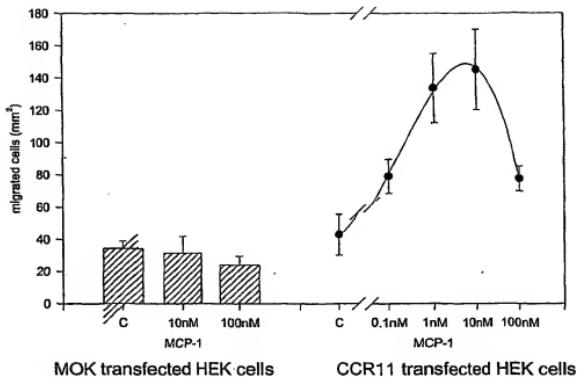
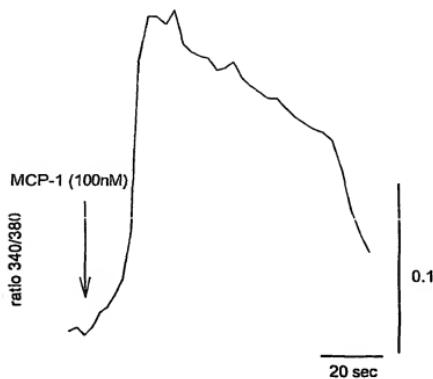


Fig. 7



A



B

C

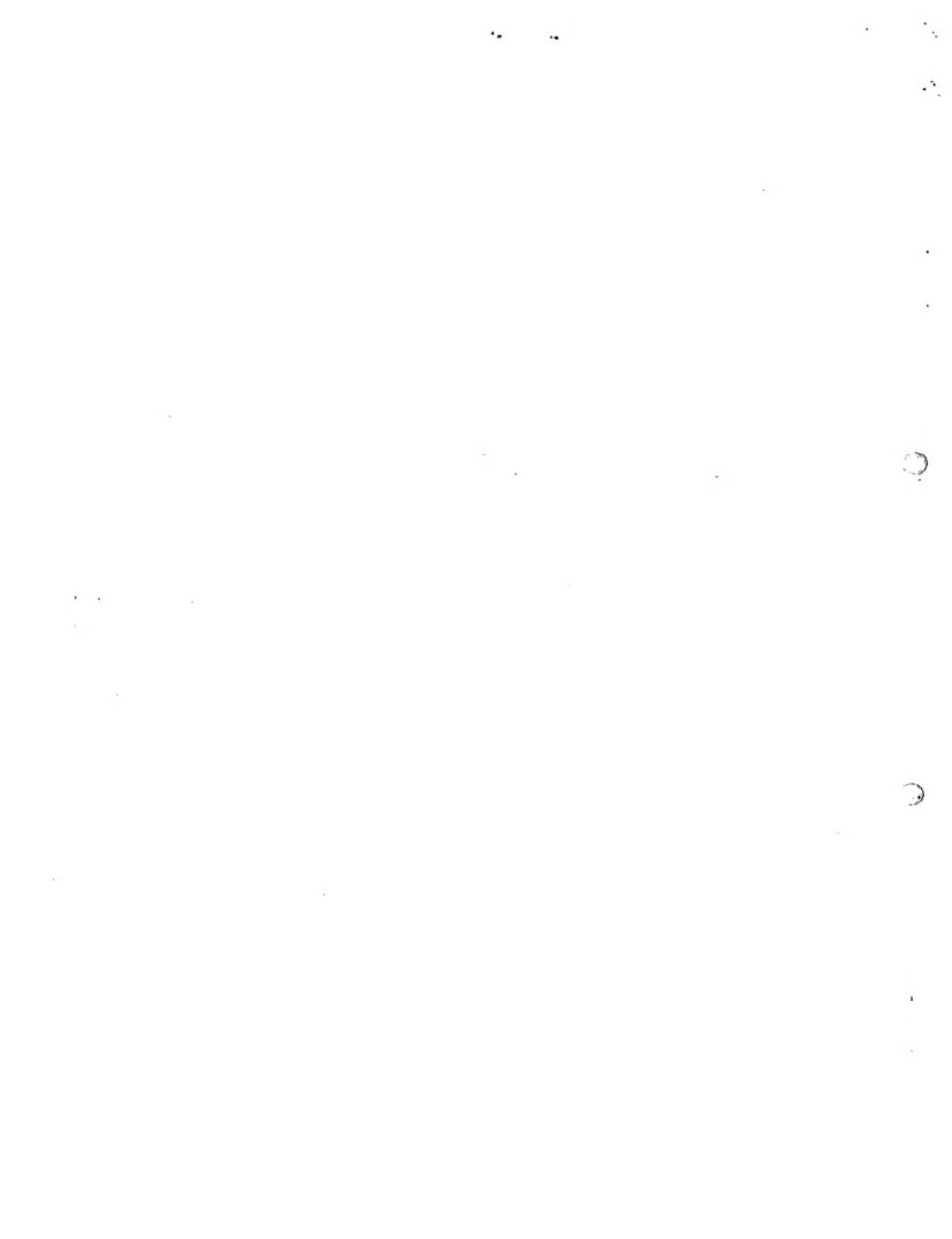
C

Fig. 8

## CELESTIAL W (1.81) multiple sequence alignment

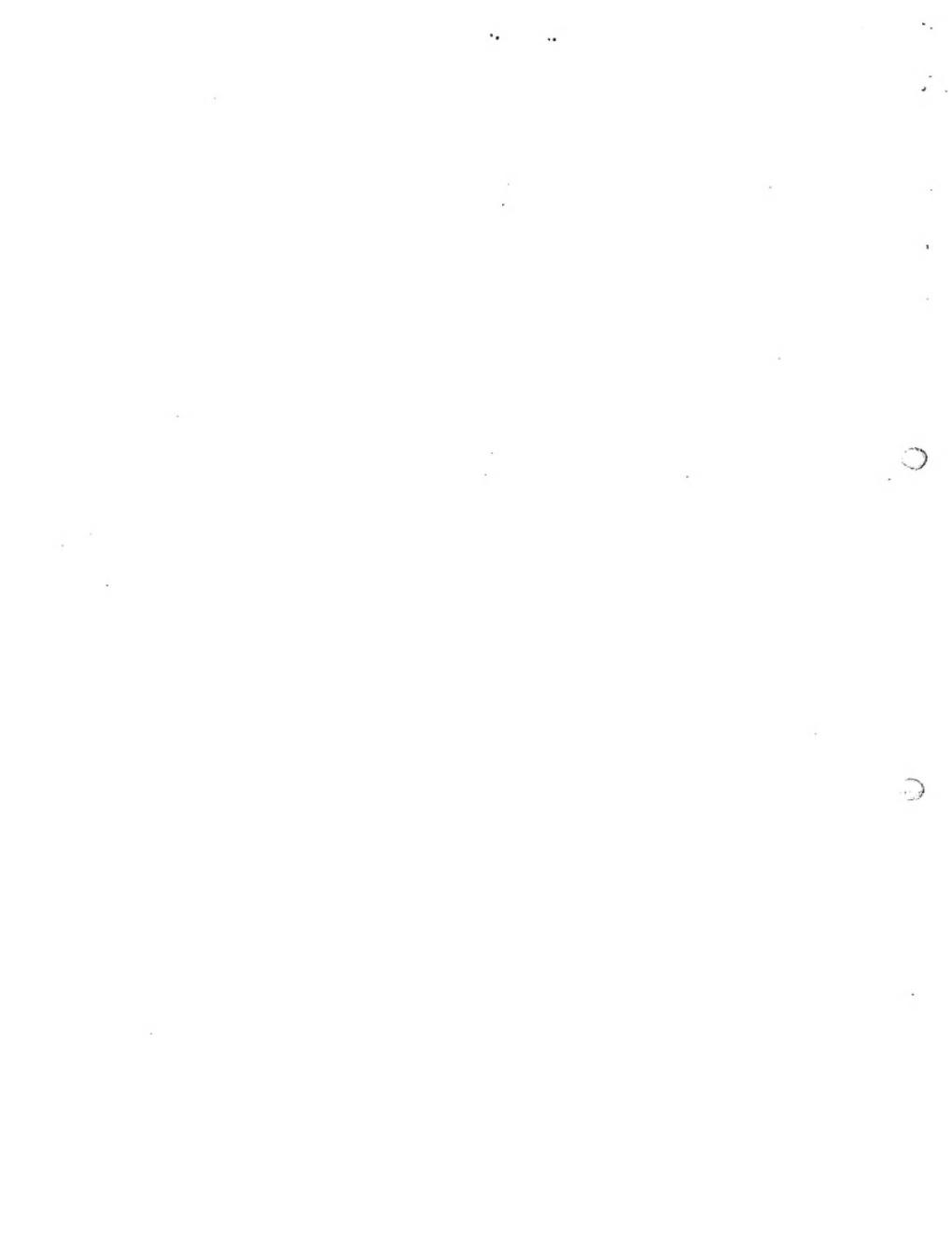


Fig. 8a



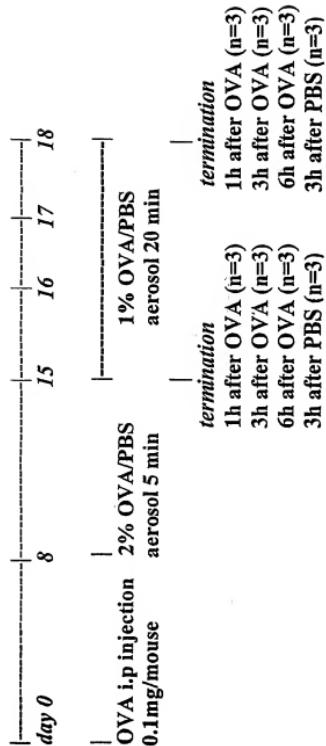
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Fig. 8b



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Fig. 9



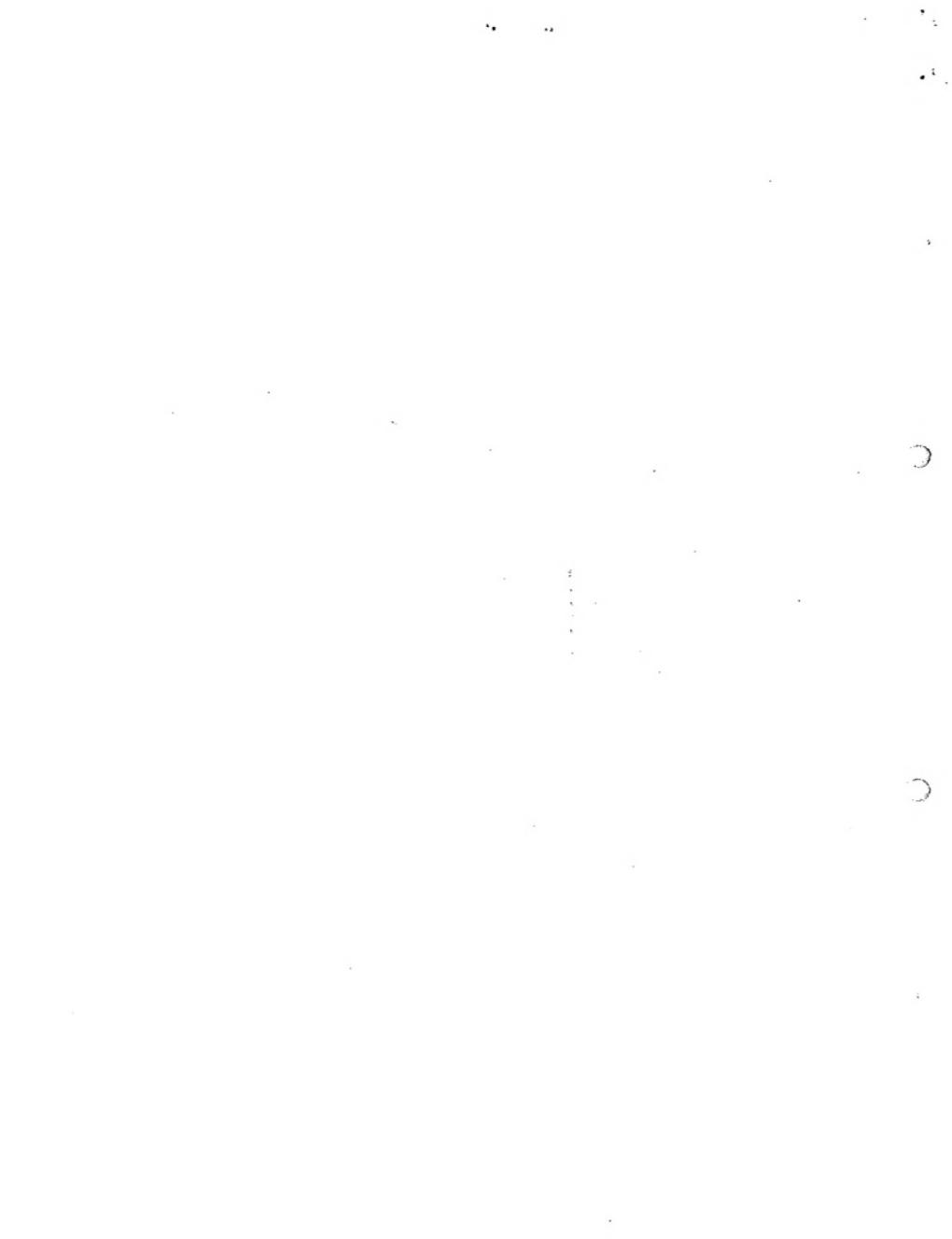
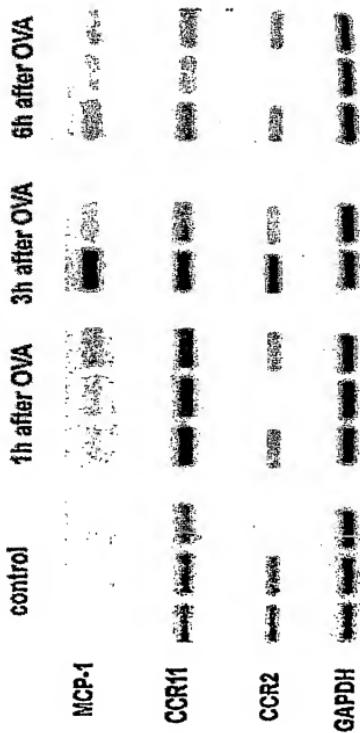


Fig. 10





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Fig. 11

